Developmental Biology **215,** 278–287 (1999)
Article ID dbio.1999.9442, available online at http://www.idealibrary.com on IDEAL®

Maspin Plays an Important Role in Mammary Gland Development

Ming Zhang,*,[1] David Magit,† Florence Botteri,‡ Heidi Y. Shi,* Kongwang He,* Minglin Li,§ Priscilla Furth,§ and Ruth Sager†

**Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030; †Dana Farber Cancer Institute, 44 Binney Street, Boston, Massachusetts 02115; ‡Friedrich Miescher Institute, Basel, Switzerland; and §University of Maryland at Baltimore, Baltimore, Maryland 21201*

Maspin is a unique member of the serpin family, which functions as a class II tumor suppressor gene. Despite its known activity against tumor invasion and motility, little is known about maspin's functions in normal mammary gland development. In this paper, we show that maspin does not act as a tPA inhibitor in the mammary gland. However, targeted expression of maspin by the whey acidic protein gene promoter inhibits the development of lobular–alveolar structures during pregnancy and disrupts mammary gland differentiation. Apoptosis was increased in alveolar cells from transgenic mammary glands at midpregnancy. However, the rate of proliferation was increased in early lactating glands to compensate for the retarded development during pregnancy. These findings demonstrate that maspin plays an important role in mammary development and that its effect is stage dependent.

Key Words: **maspin; serpin; lobular–alveolar structure; mammary development; whey acidic protein.**

INTRODUCTION

Serine protease inhibitors (serpins) comprise a large family of molecules that play a variety of physiological roles *in vivo*. Serpins can be divided into two categories: inhibitory and noninhibitory serpins. Noninhibitory serpins, typified by ovalbumin and PEDF, do not exhibit protease inhibitor activity, but rather function as a storage protein and inducer of cell differentiation, respectively (Hunt and Dayhoff, 1980; Tombran-Tink *et al.*, 1992). Inhibitory serpins ablate serine proteases through their functional domain-reactive site loop (RSL) (Potempa *et al.*, 1994). Interestingly, some inhibitory serpins have evolved other regulatory functions. For example, plasminogen activator inhibitor 1 (PAI-1) not only specifically inhibits tPA and uPA, but also regulates cell adhesion, which is independent of its protease inhibitor function, by blocking integrin $\alpha v\beta 3$ binding to vitronectin (Stefansson and Lawrence, 1996; Deng *et al.*, 1996).

Maspin is a unique member of the serpin family that shares extensive homology with PAI-1 and other serpins (Zou *et al.*, 1994). Initially identified as a class II tumor suppressor gene, maspin has been shown to inhibit invasion and motility of mammary carcinoma cells in culture (Zou *et al.*, 1994; Sheng *et al.*, 1996; Zhang *et al.*, 1997a). Tumor transfectants expressing maspin exhibit decreased growth and metastasis in nude mice (Zou *et al.*, 1994). Maspin gene expression is not detected in most breast tumors and loss of its expression is correlated with tumor invasiveness (Zhang *et al.*, 1997b). In human breast tissue, maspin is produced predominantly by myoepithelial cells and it has been suggested that these maspin-expressing cells form a defensive barrier for the progression from ductal carcinoma *in situ* to more invasive carcinomas (Sternlicht *et al.*, 1997).

Structurally, maspin has a unique RSL different from that of other inhibitory serpins (Fitzpatrick *et al.*, 1996; Hopkins and Whisstock, 1994). It has been shown that the short RSL in maspin presents a structural difficulty that would not allow maspin to undergo the stressed-to-relaxed transition, a feature characteristic of noninhibitory serpins (Pemberton *et al.*, 1995). However, a recent report by Sheng *et al.* demonstrated that maspin could specifically function as a protease inhibitor by blocking tissue plasminogen activator *in vitro* (Sheng *et al.*, 1998). This observation led to the conclusion that maspin's anti-protease activity was responsible for its ability to inhibit tumor cell invasion and

[1] To whom correspondence should be addressed at the Department of Cell Biology, Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030. Fax: (713) 798-8012. E-mail: mzhang@bcm.tmc.edu.

0012-1606/99 $30.00

motility (Sheng *et al.*, 1994, 1998). However, the biological relevance of a maspin/tPA interaction has not been confirmed *in vivo*.

The murine mammary gland undergoes cycles of growth, morphogenesis, and involution throughout development. Interactions between epithelial cells and the extracellular matrix (ECM) play an essential role in mammary gland development (Talhouck *et al.*, 1991; Sympson *et al.*, 1994). Disruption of cell adhesion disrupts normal mammary morphogenesis and differentiation (Faraldo *et al.*, 1998). Indeed, destruction of the ECM by proteases, which disrupt cell–cell and cell–basement membrane contacts, has a similar defect in mammary development (Alexander *et al.*, 1996; Sympson *et al.*, 1994; Talhouk *et al.*, 1992; Witty *et al.*, 1995). To delineate maspin's function *in vivo*, we have generated transgenic mice that overexpress maspin in the mammary gland under the control of the WAP (whey acidic protein; Hennighausen *et al.*, 1982) gene promoter. Since maspin is expressed in mammary epithelial cells, as is the WAP gene, and WAP expression begins at midpregnancy and remains on throughout lactation (Pittius *et al.*, 1988), this study assessed the effect of maspin transgene expression on normal mammary gland development during this time frame. Our data demonstrate that overexpression of maspin by the WAP promoter inhibits alveolar development and differentiation.

MATERIAL AND METHODS

Mice

For phenotypic comparisons (histology, RNA, protein), all mice in this study were age matched with wild-type littermates as controls. Virgin mice were between 7 and 8 weeks. Unless otherwise indicated, mice between 7 and 8 weeks of age were used for mating. Timed pregnancy was determined by plug appearance and confirmed by delivery. Involution was forced by removing the pups from the mother at lactation day 10 except for the RT-PCR assay, when pups were taken from mother at lactation day 21. For each time point, samples from at least two transgenic and wild-type mice were examined.

Zymogram Gel

The activity of plasminogen activators (tPA and uPA) in mammary gland was detected in casein gels supplemented with plasminogen as described by Talhouk *et al.* (1991). Fresh samples from mammary tissues were frozen in liquid nitrogen. Tissue was then pulverized into a fine powder and suspended 1:5 (wt/vol) in extraction buffer (1% Triton X-100, 500 mM Tris–HCl buffer, pH 7.6, 200 mM NaCl, 10 mM $CaCl_2$). The suspension was frozen on dry ice and thawed four times and microfuged (12,000*g* for 30 min). The supernatant was used for zymogram gel. Each sample loaded contained 15 μg protein. For casein gels, 12% polyacrylmide with 1 mg/ml purified casein (Sigma, Inc., St. Louis, MO) was prepared with plasminogen (6 μg/ml; American Diagnostica, Inc., CT). Purified single-chain tPA was purchased from American Diagnostica and 0.2 ng (0.1 IU) tPA was used per lane (commercial tPA sample contains a small amount of uPA activity). Recombinant maspin was prepared as described before (Zhang *et al.*, 1997a) and concentrated by centrifugation in Centracon (Micron, Inc., MA). The tPA present in mammary gland (15 μg extract) was quantitated by comparing its activity to that of a purified tPA control. The level of tPA in virgin or pregnant mammary gland (15 μg extract) was estimated at about 0.1 ng. To characterize the effect of maspin on plasminogen activators, mammary protein extracts or purified tPA (0.2 ng) were either incubated with concentrated maspin (2 μg in 15-μl reaction solution of 2.6 μM) or mock treated for 30 min at 37°C before mixing with 4× SDS sample buffer. The molar ratio of maspin to tPA was calculated to exceed 20,000 to 1 in reactions containing mammary extracts. After electrophoresis, the zymogram gel was incubated in a sealed plastic bag with concentrated maspin at 10 μM or as a control with PMSF at the recommended concentration of 5 mM for a period of 24 h in 50 mM Tris–HCl, 10 mM $CaCl_2$, pH 7.6. The gel was then stained with Coomassie Blue R250 for 45 min and photographed.

Whole-Mount Preparations

For whole-mount comparison, two samples from each time point were examined. Mammary gland whole mounts were performed by spreading the fourth inguinal gland on a glass slide followed by fixation in Carnoy's solution for 60 min at room temperature (Medina, 1973; Li *et al.*, 1996). Following fixation, the gland was dehydrated by 70% EtOH, followed by staining with carmine alum overnight (0.2% carmine, 0.5% aluminum sulfate). The tissue then was dehydrated and mounted on glass slides using routine methods.

Histology and Immunostaining

Mammary glands were removed under anesthesia from normal and transgenic females at different stages of development. Unless mentioned in the text, the samples were taken from left or right inguinal No. 4 mammary gland for histology. Mammary tissues were fixed in 10% neutral formalin buffer and embedded in paraffin and sectioned at 5 μm. PCNA staining was performed as recommended by the manufacturer (Zymed, Inc., San Francisco, CA). TUNEL assay was carried out as described by Li *et al.* (1995) utilizing a TDT nick end-labeling kit by Boehringer Mannheim (Boehringer Mannheim, Inc., Mannheim, Germany).

Generation of Transgenic Mice

The WAP–maspin vector (Fig. 3A) was generated by PCR amplification of the mouse maspin coding region (Zhang *et al.*, 1997a) with primers flanked by *Kpn*I and *Sal*I sites (5′-CGGTACCGGATCCATGGATGCCCTGAGACTGGCA-3′ and 5′-TCCCCCGGGTCGACTACAGACAAGTTCCCTGAGA-3′). After ligating the mouse maspin cDNA between WAP exon 1 and 3, the maspin cDNA portion was sequenced to ensure the absence of mutations. The vector was digested with *Not*I and *Hin*dIII and the lineralized WAP–maspin construct was injected into C57BL/6 × BALB/c F_1 fertilized eggs. The injected embryos were transferred into oviducts of recipient pseudo-pregnant female C57BL/6 mice, and the offspring were analyzed by Southern blot. Six transgenic founders were generated and crossed with C57BL/6 mice to generate F_1 lines. Two of the best expressing lines of maspin transgenics were established for further analysis, and positive litters were screened by PCR analysis using the following primers. Endogenous maspin (199 bp): 5′ primer, GATGGTGGTGAGTCCATC; 3′ primer, TC-

CCCCGGGTCGACTACAGACAAGTTCCCTGAGA. Transgene (218 bp): 5′ primer, GATGGTGGTGAGTCCATC; 3′ primer, GCTCTAGAGGTGTACATGTCATGACACAGTCGAC.

For RT-PCR identification of transgene and endogenous maspin mRNAs (RT-PCR is not used to quantitate the difference of expression level in this case), the 3′ primers of transgene and endogenous maspin above were used for reverse transcription. Both transgene and endogenous maspin products were PCR amplified with the above sets of primers. PCR was carried out for 35 cycles in two sets of identical reaction conditions (95°C for 1 min, 52°C for 1 min, 72°C for 1 min 30 s, 34 cycles).

Northern Blot Analysis

RNA was isolated by guanidine as previously described (Swisshelm *et al.*, 1994). Total RNA was fractionated on 1% agarose–1.7 M formaldehyde gels, transferred to a Zetaprobe (Bio-Rad Laboratories, Richmond, CA) membrane in 20× SSC, and baked for 1 h at 80°C. Blots were probed with a 1.5-kb *Eco*RI/*Xho*I fragment from the mouse maspin cDNA plasmid. Ribosomal protein gene 36B4 was used as an internal loading and transfer control (Laborda, 1991).

Western Blot Analysis

The mouse mammary tissues were frozen in liquid N_2 and lysed in electrophoresis sample buffer (Zou *et al.*, 1994; Zhang *et al.*, 1997a), and the extracts were subjected to electrophoresis on a 10% SDS–polyacrylamide gel and transferred to Immobilon membrane. For β-casein and WAP protein analysis, 20 μg of whole extract for each sample was loaded for transfer. The blot was exposed first to anti-WAP antibody and reexposed to anti-β-casein antibody. For anti-WAP antibody, a 1:1000 dilution was used and followed by exposure to a 1:2500 dilution of peroxidase-conjugated goat anti-rabbit IgG polyclonal antibody (Amersham, Arlington Heights, IL) as described (Li *et al.*, 1996). Anti-rat β-casein monoclonal antibody (kindly provided by Dr. Jeff Rosen's laboratory) was originally a gift from Dr. Mina Bissell. A dilution of 1:2500 was used for this antibody followed by an exposure to a 1:2500 dilution of sheep anti-mouse IgG (HRP conjugated) (Amersham). The blot was finally probed with an anti-β-actin monoclonal antibody (Sigma) at a 1:2500 dilution followed by an exposure to a 1:10,000 sheep anti-mouse IgG (HRP conjugated) (Amersham).

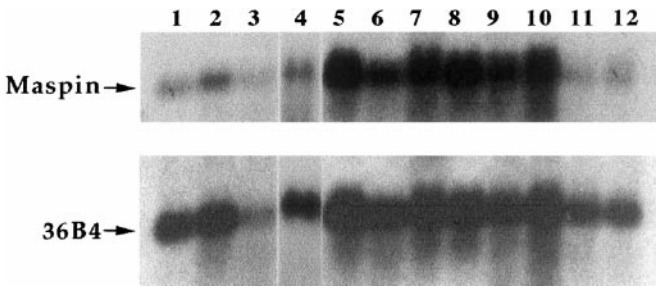


FIG. 1. Northern blot analysis of maspin gene in normal mouse mammary glands during development. Each lane contains 10 μg of total RNAs. Samples are from virgin (lanes 1, 2), day 10 pregnant (lanes 3, 4), day 19 pregnant (lanes 5, 6), day 3 lactation (lanes 7, 8), day 3 involution (lanes 9, 10), and day 10 involution (lanes 11, 12). The blot was hybridized with a cDNA probe for mouse maspin. The ribosomal protein gene 36B4 was used as a loading and transfer control.

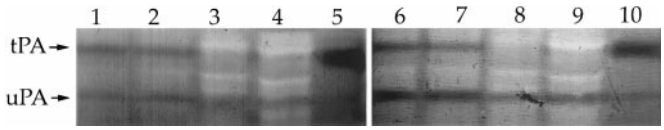


FIG. 2. The effect of maspin on plasminogen activator activity during mammary gland cycles assayed by SDS–substrate zymography. Mammary samples from 8-week-old virgin (lanes 1, 6), day 15 pregnancy (lanes 2, 7), day 3 lactation (lanes 3, 8), day 3 involution (lanes 4, 9), and pure tPA (lanes 5, 10) were assayed in casein/plasminogen gel. Lanes 6–10 were duplicate samples of lanes 1–5 loaded on the same gel. Samples in lanes 6–10 were pretreated with maspin at 2.6 μM and the gel was continuously incubated after electrophoresis with maspin at 10 μM as described under Material and Methods. All lanes were loaded with 15 μg of mammary extracts or 0.2 ng of tPA control.

RESULTS

Maspin Does Not Act as a tPA Inhibitor in Mammary Gland

To determine the temporal expression of maspin during mammary gland development, Northern blot assays were performed (Fig. 1). Maspin mRNA was expressed at relatively low levels in virgin and early pregnant mammary glands; however, maspin gene expression increased during late pregnancy and lactation. Following involution, maspin mRNA levels decreased to those observed in the prepregnant stage (lanes 11 and 12).

To delineate whether maspin regulated protease activity in the mammary gland, SDS–PAGE containing casein and plasminogen was carried out using mammary samples obtained at different stages of development. The activities of 48- (uPA) and 68-kDa (tPA) were observed in casein/plasminogen gels as previously reported (Talhouk *et al.*, 1991; Lund *et al.*, 1996) (Fig. 2). The tPA activity was high in virgin and pregnancy and low in lactation but began to elevate 3 days after forced involution. It was estimated that the mammary gland (15 μg extract from virgin or pregnant stage) contained less than 0.1 ng tPA (see Material and Methods). As shown in Fig. 2, pretreatment of mammary extracts or pure tPA with maspin at 2.6 μM (molar ratio of maspin to tPA exceeded 20,000 to 1) and continuous incubation of the zymogram gel with maspin at 10 μM did not inhibit the activity of tPA or uPA. However, recombinant maspin used was functional against cell motility at submicromolar concentration (data not shown). As a positive control, treatment of the gel by PMSF, a nonspecific serine protease inhibitor, effectively abolished tPA and uPA activity (data not shown).

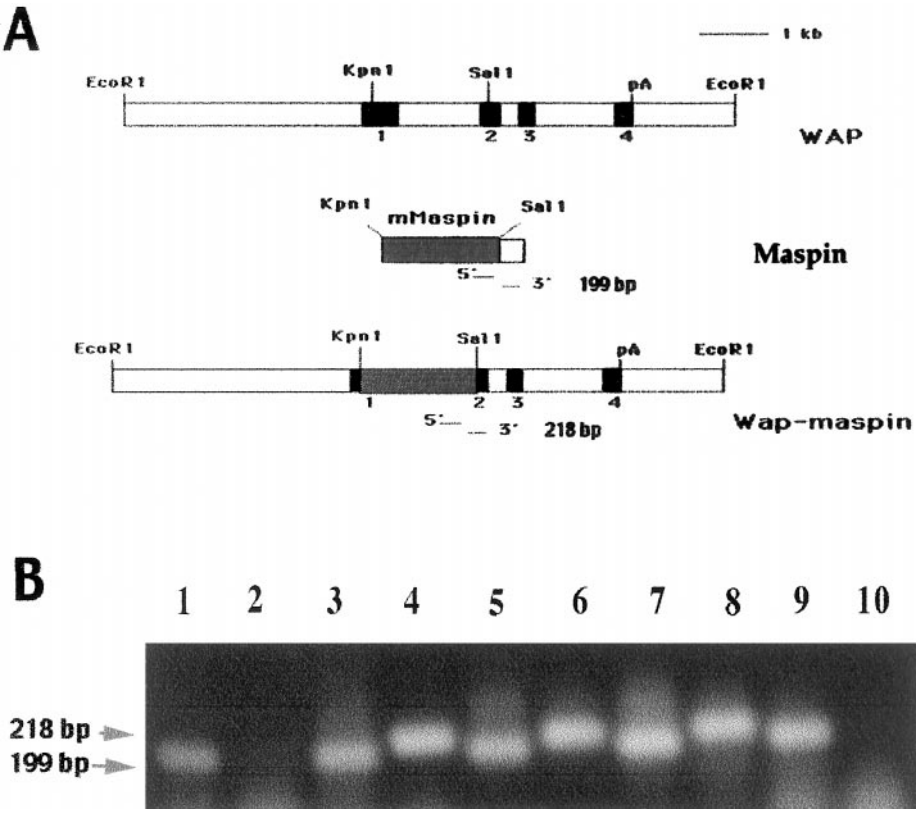


FIG. 3. WAP–maspin transgenic mice. (A) Schematic diagram of the structure of the WAP–maspin construct. WAP genomic DNA was digested by *Kpn*I and *Sal*I to remove exons 1 and 2 and intron 1. The mouse maspin (mMaspin) cDNA (gray box) was digested with *Kpn*I and *Sal*I and ligated to the digested WAP plasmid to create the WAP–maspin construct. (B) Expression of maspin in the mammary glands of transgenic mice. RNAs (2 μg) from 8-week-old virgin (1, 6), day 15 of pregnancy (2, 7), day 19 of pregnancy (3, 8), day 3 of lactation (4, 9), and day 4 of involution (5, 10) samples were analyzed by RT-PCR with two sets of primers (see Material and Methods). Samples of odd number (1, 3, 5, 7, 9) gave rise to an endogenous maspin PCR product of 199 bp, while samples of even number (2, 4, 6, 8, 10) yielded a transgene transcript of 218 bp.

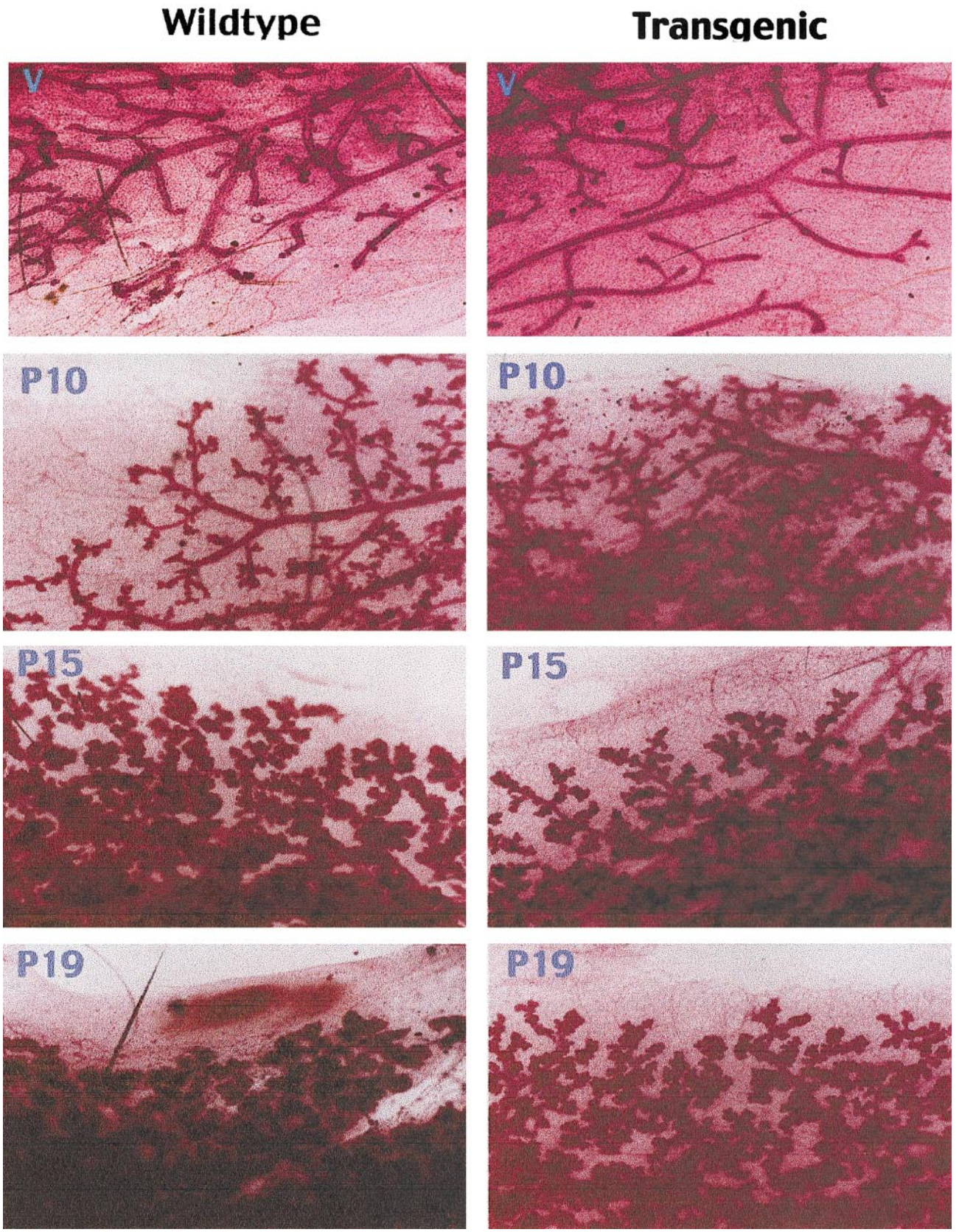
WAP–Maspin Transgenic Mice

To further investigate the role of maspin in mammary development, we utilized a transgenic mouse system to examine the effect of overexpression of maspin under control of the WAP promoter. The whey acidic protein has been shown to be exclusively expressed in mammary epithelial cells during midpregnancy and lactation.

Transgenic mice were generated by injecting embryos with the construct as shown in Fig. 3A. Screening of founders was performed by Southern blot analysis using DNA isolated from mouse tissues (data not shown). Of the six founder lines generated, two lines that displayed similar phenotypes were maintained and the results reported below were obtained from samples of the same line.

The expression of both endogenous maspin and the transgene in transgenic mouse mammary glands was examined qualitatively by reverse transcription-PCR (Fig. 3B). RNA was isolated from the mammary glands of transgenic mice at different stages of development. As shown in Fig. 3B, the PCR product of the transgene differed from that of endogenous maspin by 19 bp (218 bp vs 199 bp), allowing them to be distinguished by electrophoresis. As previously

FIG. 4. Whole-mount analysis of normal and transgenic mammary glands during development. Inguinal No. 4 glands were taken for all analyses. The virgin mammary glands (V) were from age-matched virgin transgenic and wild-type mice at 8 weeks of age. Age-matched mice were mated at 7 weeks of age for pregnancy. P10, 15, and 19 indicate days 10, 15, and 19 of pregnancy. Photographs were taken with a 4× power objective.



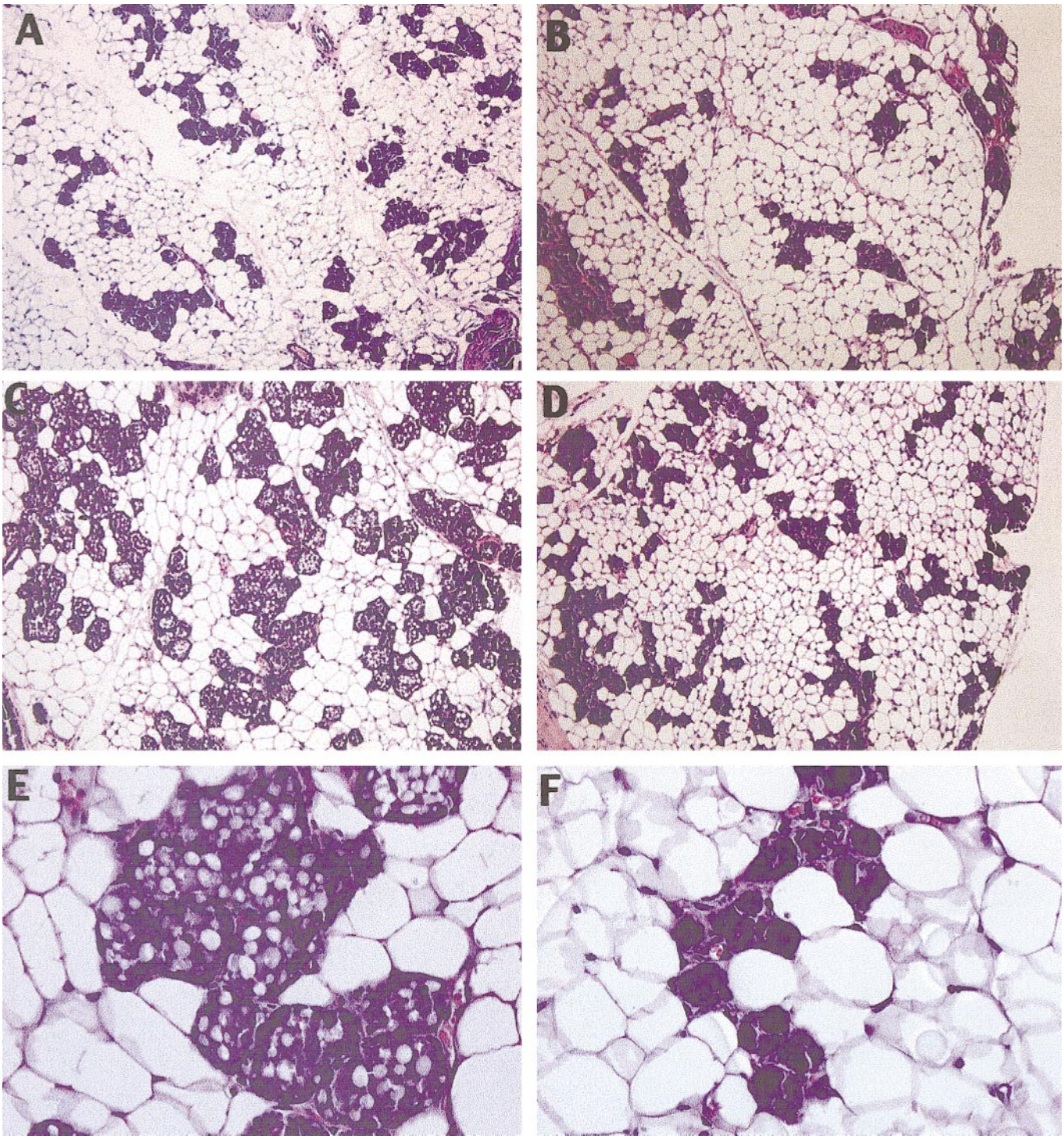


FIG. 5. Histological analysis of mammary tissues from the following mice: (A) wildtype at day 15 pregnancy, (B) transgenics at day 15 pregnancy, (C) wildtype at day 19 pregnancy, and (D) transgenics at day 19 pregnancy. (E and F) (Same magnification) High-power pictures from (C) and (D), respectively. Note the reduced numbers of alveolar structures and the smaller lumen size in the transgenics (D, F). Photographs were taken with a 10× objective for (A–D) and with a 40× objective for (E and F).

shown by Pittius *et al.* (1988), the WAP–maspin transgene was expressed during late pregnancy and lactation, but was not detectable in virgin mice and glands undergoing involution (Fig. 3B).

To characterize the mammary gland phenotype of the WAP–maspin mice, whole-mount preparations of the mammary glands from wild-type and transgenic mice were analyzed (Fig. 4). Ductal elongation and branching appeared to be normal in wild-type and transgenic virgin mice (Fig. 4). As shown in Fig. 4, no differences in alveolar structures were present between the transgenic and wild-type animals up to day 10 of pregnancy; however, minor differences did become noticeable at day 15 of pregnancy following the activation of the WAP promoter-driven transgene. These mammary glands exhibited decreased alveolar densities, which were further reduced compared to controls at day 19 and resembled the morphology of the midpregnant wild-type controls.

This defect was also observed in histological samples taken from mid- to late-pregnant transgenic mammary glands (Fig. 5). A minor reduction in the numbers of lobular–alveolar structures was noted at day 15 of pregnancy in the transgenic line (Figs. 5A and 5B). The alveolar units in wild-type glands contained large lumens that were filled with fat droplets at day 19 of pregnancy (Fig. 5C). In contrast, the mammary glands from transgenic mice contained fewer lobular–alveoli structures, and the size of each alveolar structure was greatly reduced (Figs. 5D, 5E, and 5F). In many cases, the lumens of the alveoli were closed. This defect was due to the expression of the transgene activated by the WAP promoter from midpregnancy.

Increased Apoptosis and Proliferation in Midpregnant and Early Lactating Mammary Gland of Transgenic Mice

Since the underdevelopment of the mammary glands in the WAP–maspin transgenic mice could have arisen from either decreased proliferation or increased apoptosis or a combination of the two, TUNEL and PCNA immunohistochemistry assays were carried out utilizing pregnant and early lactating mammary glands from wild-type and transgenic mice. As shown in Table 1, the apoptotic rate was significantly increased in transgenic glands at midpregnancy ($2.29 \pm 0.26\%$) compared to controls ($0.91 \pm 0.09\%$). In contrast, little difference was observed in cell proliferation at day 15 of pregnancy (Table 2). However, during lactation, the apoptosis and proliferation profiles changed significantly in the transgenic strain. Secretive alveolar cells occupied the majority of the fat pad and there was a low rate of proliferation and apoptosis in samples taken from normal mammary glands. This observation contrasted to the results obtained from the WAP–maspin mice, in which a large percentage of the fat pad was devoid of alveolar cells and an increased rate of proliferation was observed (Table 2). Although apoptosis was still higher in the transgenic glands than in normal control mice, there was a net increase in the number of alveolar cells between day 1 and day 10 lactation samples (data not shown). Both proliferation and apoptosis index decreased quickly as lactation proceeded and by lactation day 10, very few cells were PCNA-positive and apoptotic in both transgenic and normal mice.

Effect of Transgene Expression on Milk Gene Expression

The defect in alveolar structures in the WAP–maspin mice during late pregnancy severely hampered the ability of the mother to successfully nurse her entire litter. Indeed, most of the pups died due to insufficient milk production. However, these pups could be rescued by fostering them to a BALB/c nontransgenic lactating female. The number of pups that a transgenic mother could nurse varied between animals. A survey of five sibling mothers at their first pregnancy yielded an average survival rate of 3.6 pups/litter.

Since milk protein genes can function as differentiation markers for the mammary gland, we compared their expression patterns in transgenic and wild-type control mice. Western blot analysis showed that WAP and β-casein were highly expressed in wild-type mammary glands at day 19 of pregnancy and during lactation (Fig. 6). However, WAP and β-casein were not detectable in day-19 pregnant transgenic mice (lanes 8 and 1). Both milk proteins were present in lactating day 1 transgenic glands, but at a reduced levels, which increased as lactation progressed (lanes 6 and 5). This observed decrease was likely due to the effect of reduced number of alveolar cells and closed lumens in the late-pregnant transgenic mice.

DISCUSSION

We have demonstrated that maspin plays an important role in mammary gland development. Targeted expression of maspin, via the WAP promoter, inhibits alveolar development by increasing apoptosis and disrupts the process of differentiation. Since the transgene encodes the exact same maspin protein as the endogenous gene in the same cells, the phenotype is attributed directly to WAP–maspin overexpression. The phenotype clearly depends upon the level of transgene expressed, as not all of the transgenic lines exhibit the same degree of alveolar disruption, which correlates to WAP–maspin overexpression. Even though the line tested displayed the most severe phenotype, due to the patchy expression pattern of the WAP transgene (Sympson *et al.*, 1994), there are variations in alveolar structure, rate of proliferation, and apoptosis (Tables 1 and 2). This also resulted in decreased expression of endogenous maspin. In some late-pregnant mammary glands that were severely underdeveloped in the transgenic mice, overall maspin levels were down because of the drastic decrease of epithelial cells via increased apoptosis. Therefore, we did not use total maspin protein as a marker in our characterization.

TABLE 1

The Rate of Apoptosis in Wildtype and Transgenic Mice

	WT	Transgenic	*P* value
15-day pregnant	0.91 ± 0.09 (3)	2.29 ± 0.26 (3)	$P < 0.01$
19-day pregnant	0.54 ± 0.05 (2)	0.91 ± 0.54 (3)	$P < 0.04$
Day 1 lactation	0.15 ± 0.05 (2)	0.79 ± 0.11 (3)	$P < 0.01$

Note. Values are presented as percentages of apoptotic cells (means ± SD). Number of animals analyzed is indicated in parentheses. About 1000–1200 cells per sample were counted. Statistical analysis was done by the Student *t* test.

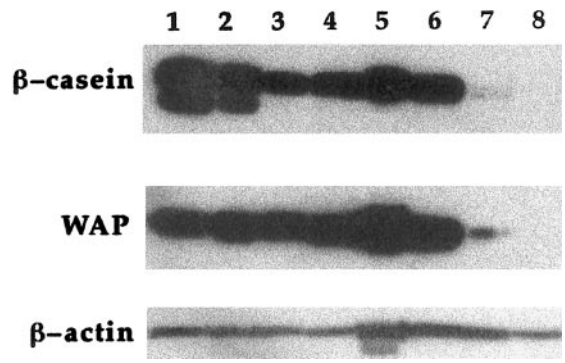


FIG. 6. Western blot analysis of milk proteins in the mammary glands of transgenic and nontransgenic C57BL/6 mice. Lanes 1–4 were from C57BL/6 mammary tissues and lanes 5–8 were from transgenic mice. Age-matched samples used were from day 19 of pregnancy (lanes 1, 8), day 1 of lactation (lanes 2, 7), day 3 of lactation (lanes 3, 6), and day 10 of lactation (lanes 4, 5). Aliquots of whole-cell extracts (20 μg) were loaded on each lane and separated by 10% SDS–PAGE and transferred to PVDF membrane. The blot was probed with antibodies against WAP, β-casein, and β-actin. β-Actin was used as a loading and transfer control.

Maspin Does Not Act as a tPA Inhibitor in Mammary Gland

Previous biochemical analysis demonstrated that maspin could interact with single-chain tissue plasminogen activator and inhibit the conversion of plasminogen to plasmin with a K_i value of 0.13 μM (Sheng *et al.*, 1998). Under similar assay conditions, PAI-1 and PAI-2 also inhibited tPA with a value either smaller than (PAI-1, 0.033 μM) or similar to (PAI-2, 0.55 μM) maspin (Sheng *et al.*, 1998). It was first suspected that maspin might act as a tPA inhibitor during mammary gland development. However, zymography assay has excluded this possibility. In theory, inhibitory serpin generally binds to its target protease in 1:1 stoichiometry, inactivating the protease by forming a complex stable to heat or SDS denaturation (Potempa *et al.*, 1994; Pemberton *et al.*, 1995). During the time of pretreatment, the exogenous maspin was in such an excess (20,000:1) that we expected that it would at least decrease the tPA activity should tPA be neutralized by maspin. However, this treatment and further incubation of the zymogram gel for 24 h with maspin at 10 μM or about 80× K_i did not have any detectable inhibition of tPA activity. We also compared the tPA activity of transgenic and wildtype mammary glands at various stages of mammary development including midpregnancy and lactation by casein zymography. No significant difference in tPA activity was observed (data not shown). Therefore, maspin's mechanism of action is unlikely to be mediated through tPA inhibition in the mammary gland. However, we cannot rule out the possibility that maspin may inhibit other unidentified proteases in mammary gland. Alternatively, maspin may possess additional functions in mammary gland. We have recently found out that maspin acts as an angiogenesis inhibitor, and this action is independent of the RSL domain of maspin (Zhang *et al.*, in review). It becomes more clear that the multifunctionality is a common feature for maspin and other serpins (Bajou *et al.*, 1998; Deng *et al.*, 1996).

Similar to studies that have been reported for PAI-1, we believe that maspin may primarily regulate cell adhesion and motility in mammary cells, possibly by regulating integrin profiles. Indeed, expression of a dominant-negative β1 integrin in the mammary gland, which disrupted the function of β1 and its associated integrins, resulted in a phenotype similar to that observed in the WAP–maspin transgenic mice (Faraldo *et al.*, 1998). Both maspin and chimeric β1 transgene expression caused underdevelopment of the mammary gland in midpregnancy and early lactation, which was accompanied by an increase in apoptosis. In early lactation, milk protein levels were also reduced. The similarity in phenotypes suggests that overexpression of maspin may act to perturb integrin regulation or other associated cell adhesion molecules. This hypothesis is partially supported by an *in vitro* study which showed that exogenous maspin modified cell attachment to fibronection by regulating integrin profiles, including down-regulation of β1 integrin (Seftor *et al.*, 1998). However, there are some differences in the observed phenotype between these two transgenic mice strains. For example, the proliferation of alveolar cells in the β1-transgenic mice decreases during pregnancy, while maspin transgenic mice

TABLE 2

The Rate of Proliferation in Wildtype and Transgenic Mice

	WT	Transgenic	*P* value
15-day pregnant	11.73 ± 1.93 (4)	10.50 ± 2.59 (4)	$P < 0.89$
Day 1 lactation	2.73 ± 1.72 (3)	9.49 ± 1.86 (3)	$P < 0.01$

Note. Values are presented as percentages of PCNA-positive nuclei (means ± SD). Number of animals analyzed is indicated in parentheses. In all cases, 1000–1200 cells per sample were counted. Statistical analysis was done by the Student *t* test.

showed no change. Since alterations in integrin profiles, especially the dimerization of subunits, are not well characterized in the mammary gland, it is not known whether other integrins or cell adhesion molecules are regulated by maspin.

Disruption of the Process of Differentiation at Midpregnancy

During pregnancy, alveolar cells proliferate and differentiate. By parturition, the mammary gland terminally differentiates and functions mainly to secrete milk for nursing the progeny. Milk protein genes, such as WAP and β-casein, are hallmarks of this process. In the WAP–maspin transgenic mice, we observed that WAP and β-casein gene expression was diminished from late pregnancy to early lactation, demonstrating that the differentiation process is disrupted. This is reflected by the fact that the lumen of the transgenic gland is reduced in size and closed during late pregnancy compared to wild-type controls. This phenotype correlates with other *in vivo* and *in vitro* studies, suggesting that the appropriate cell adhesion interactions between the extracellular matrix and epithelial cells are required for milk gene expressions (Alexander *et al.*, 1996; Streuli *et al.*, 1995; Talhouk *et al.*, 1992).

Increased Apoptosis in Pregnancy and Proliferation in Lactation in Transgenic Mammary Gland

We have shown that WAP–maspin transgene expression significantly increases the rate of apoptosis in midpregnancy, while the proliferation rate was largely unchanged. The mechanism by which maspin regulates apoptosis is not known. One possibility is that overexpression of maspin perturbs the adhesion of alveolar cells to the ECM as does the chimeric β1 transgene and thus inhibits the motility of alveolar cells at a stage when invasion into the fat pad is critical. The proliferating alveolar cells are unable to migrate out, leading to increased apoptosis and a resulting small lumen. This is consistent with the concept that proper interactions of mammary epithelial cells with the basement membrane are essential for cell survival, and their disruption will trigger signals leading to apoptosis (Frisch and Francis, 1994; Frisch and Ruoslahti, 1997).

During early lactation, the apoptotic rate in the WAP–maspin mice is relatively higher than in wildtype, but is lower than at midpregnancy. Proliferation is maintained at a high level, resulting in a net increase of alveolar cells. Another finding is that the lumen, which is closed in late pregnancy, opens following parturition. The lumen is likely opened in response to suckling (Li *et al.*, 1997). However, it is not clear why there is such an increase of proliferation in early lactation. In the normal gland, alveoli encompass the majority of the mammary fat pad by late pregnancy and are under the influence of estrogen and progesterone (Vonderhaar *et al.*, 1984). There is a transient surge of alveolar proliferation during early lactation, which allows these alveoli to engorge the entire gland (Vonderhaar *et al.*, 1988). In the maspin transgenic mice, a compensatory proliferation of epithelial cells was observed to compensate for the incomplete alveolar development during pregnancy in order to fulfill the physiological requirement to nurse the young.

In summary, we have demonstrated that maspin does not act to inhibit tPA, but may act primarily as a cell adhesion and motility regulator in mammary epithelial cells. To confirm this hypothesis, we are currently generating mice null for the maspin gene and assaying its role in normal mammary gland development. By delineating maspin's mechanism of action *in vivo* the role of maspin as a tumor suppressor can be elucidated.

ACKNOWLEDGMENTS

This paper is dedicated to the memory of Dr. Ruth Sager. The authors thank Dr. Jeff Rosen for advice and equipment support and Dr. Weston Porter for critically reading the manuscript. This work was supported in part by a DOD grant (DAMD179818029) to M.Z.

REFERENCES

Alexander, C. M., Howard, E. W., Bissell, M. J., and Werb, Z. (1996). Rescue of mammary epithelial cell apoptosis and entactin degradation by a tissue inhibitor of metalloproteinases-1 transgene. *J. Cell Biol.* **135,** 1669–1677.

Bajou, K., Noel, A., Gerard, R. D., Masson, V., Brunner, N., Holst-Hansen, C., Skobe, M., Fusenig, N. E., Carmeliet, P., Collen, D., and Foidart, J. M. (1998). Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat. Med.* **4,** 923–928.

Deng, G., Curriden, S. A., Wang, S., Rosenberg, S., and Loskutoff, D. J. (1996). Is plasminogen activator inhibitor-1 the molecular switch that governs urokinase receptor-mediated cell adhesion and release? *J. Cell Biol.* **134,** 1563–1571.

Faraldo, M. M., Deugnier, M. A., Lukashev, M., Thiery, J. P., and Glukhova, M. A. (1998). Perturbation of beta1-integrin function alters the development of murine mammary gland. *EMBO J.* **17,** 2139–2147.

Fitzpatrick, P. A., Wong, D. T., Barr, P. J., and Pemberton, P. A. (1996). Functional implications of the modeled structure of maspin. *Protein Eng.* **9,** 585–589.

Frisch, S. M., and Francis, H. (1994). Disruption of epithelial cell–matrix interactions induces apoptosis. *J. Cell Biol.* **124,** 619–626.

Frisch, S. M., and Ruoslahti, E. (1997). Integrins and anoikis. *Curr. Opin. Cell Biol.* **9,** 701–706.

Hennighausen, L. G., and Sippel, A. E. (1982). Characterization and cloning of the mRNAs specific for the lactating mouse mammary gland. *Eur. J. Biochem.* **125,** 131–141.

Hopkins, P. C., and Whisstock, J. (1994). Function of maspin. *Science* **265,** 1893–1894. [Letter; comment].

Hunt, L. T., and Dayhoff, M. O. (1980). A surprising new protein superfamily containing ovalbumin, antithrombin-III, and α1-proteinase inhibitor. *Biochem. Biophys. Res. Commun.* **95,** 864–871.

Laborda, J. (1991). 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein PO. *Nucleic Acids Res.* **19,** 3998.

Li, B., Kittrell, F. S., Medina, D., and Rosen, J. M. (1995). Delay of dimethylbenz[*a*]anthracene-induced mammary tumorigenesis in transgenic mice by apoptosis induced by an unusual mutant p53 protein. *Mol. Carcinog.* **14,** 75–83.

Li, M., Hu, J., Heermeier, K., Hennighausen, L., and Furth, P. A. (1996). Expression of a viral oncoprotein during mammary gland development alters cell fate and function: Induction of p53-independent apoptosis is followed by impaired milk protein production in surviving cells. *Cell Growth Differ.* **7,** 3–11.

Li, M., Liu, X., Robinson, G., Bar-Peled, U., Wagner, K. U., Young, W. S., Hennighausen, L., and Furth, P. A. (1997). Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proc. Natl. Acad. Sci. USA* **94,** 3425–3430.

Lund, L. R., Romer, J., Thomasset, N., Solberg, H., Pyke, C., Bissell, M. J., Dano, K., and Werb, Z. (1996). Two distinct phases of apoptosis in mammary gland involution: Proteinase-independent and -dependent pathways. *Development* **122,** 181–193.

Medina, D. (1973). Preneoplastic lesions in mouse mammary tumorigenesis. *In* "Methods in Cancer Research" (H. Busch, Ed.), pp. 3–53. Academic Press, New York.

Pemberton, P. A., Wong, D. T., Gibson, H. L., Kiefer, M. C., Fitzpatrick, P. A., Sager, R., and Barr, P. J. (1995). The tumor suppressor maspin does not undergo the stressed to relaxed transition or inhibit trypsin-like serine proteases. Evidence that maspin is not a protease inhibitory serpin. *J. Biol. Chem.* **270,** 15832–15837.

Pittius, C. W., Sankaran, L., Topper, Y. J., and Hennighausen, L. (1988). Comparison of the regulation of the whey acidic protein gene with that of a hybrid gene containing the whey acidic protein gene promoter in transgenic mice. *Mol. Endocrinol.* **2,** 1027–1032.

Potempa, J., Korzus, E., and Travis, J. (1994). The serpin superfamily of proteinase inhibitors: Structure, function, and regulation. *J. Biol. Chem.* **269,** 15957–15960.

Seftor, R. E., Seftor, E. A., Sheng, S., Pemberton, P. A., Sager, R., and Hendrix, M. J. (1998). Maspin suppresses the invasive phenotype of human breast carcinoma. *Cancer Res.* **58,** 5681–5685.

Sheng, S., Carey, J., Seftor, E. A., Dias, L., Hendrix, M. J., and Sager, R. (1996). Maspin acts at the cell membrane to inhibit invasion and motility of mammary and prostatic cancer cells. *Proc. Natl. Acad. Sci. USA* **93,** 11669–11674.

Sheng, S., Pemberton, P. A., and Sager, R. (1994). Production, purification, and characterization of recombinant maspin proteins. *J. Biol. Chem.* **269,** 30988–30993.

Sheng, S., Truong, B., Fredrickson, D., Wu, R., Pardee, A. B., and Sager, R. (1998). Tissue-type plasminogen activator is a target of the tumor suppressor gene maspin. *Proc. Natl. Acad. Sci. USA* **95,** 499–504.

Stefansson, S., and Lawrence, D. A. (1996). The serpin PAI-1 inhibits cell migration by blocking integrin alpha V beta 3 binding to vitronectin. *Nature* **383,** 441–443. [See comments]

Sternlicht, M. D., and Barsky, S. H. (1997). The myoepithelial defense: A host defense against cancer. *Med. Hypotheses* **48,** 37–46.

Streuli, C. H., Schmidhauser, C., Bailey, N., Yurchenco, P., Skubitz, A. P., Roskelley, C., and Bissell, M. J. (1995). Laminin mediates tissue-specific gene expression in mammary epithelia. *J. Cell Biol.* **129,** 591–603.

Swisshelm, K., Ryan, K., Lee, X., Tsou, H. C., Peacocke, M., and Sager, R. (1994). Down-regulation of retinoic acid receptor beta in mammary carcinoma cell lines and its up-regulation in senescing normal mammary epithelial cells. *Cell Growth Differ.* **5,** 133–141.

Sympson, C. J., Talhouk, R. S., Alexander, C. M., Chin, J. R., Clift, S. M., Bissell, M. J., and Werb, Z. (1994). Targeted expression of stromelysin-1 in mammary gland provides evidence for a role of proteinases in branching morphogenesis and the requirement for an intact basement membrane for tissue-specific gene expression. *J. Cell Biol.* **125,** 681–693. [Published erratum appears in *J. Cell Biol.*, 1996, **132,** following 752]

Talhouk, R. S., Bissell, M. J., and Werb, Z. (1992). Coordinated expression of extracellular matrix-degrading proteinases and their inhibitors regulates mammary epithelial function during involution. *J. Cell Biol.* **118,** 1271–1282.

Talhouk, R. S., Chin, J. R., Unemori, E. N., Werb, Z., and Bissell, M. J. (1991). Proteinases of the mammary gland: Developmental regulation in vivo and vectorial secretion in culture. *Development* **112,** 439–449.

Tombran-Tink, J., Li, A., Johnson, M. A., Johnson, L. V., and Chader, G. J. (1992). Neurotrophic activity of interphotoreceptor matrix on human Y79 retinoblastoma cells. *J. Comp. Neurol.* **317,** 175–186.

Vonderhaar, B. (1984). Hormones and growth factors in mammary gland development. *In* "Control of Cell Growth and Proliferation" (C. M. Veneziale, Ed.), pp. 11–33. Van Nostrand Reinhold, New York.

Vonderhaar, B. (1988). Regulation of development of the normal mammary gland by hormones and growth factors. *In* "Breast Cancer: Cellular and Molecular Biology" (M. E. Lippman and R. B. Dickson, Eds.), pp. 251–265. Kluwer Academic, Boston.

Witty, J. P., Wright, J. H., and Matrisian, L. M. (1995). Matrix metalloproteinases are expressed during ductal and alveolar mammary morphogenesis, and misregulation of stromelysin-1 in transgenic mice induces unscheduled alveolar development. *Mol. Biol. Cell* **6,** 1287–1303.

Zhang, M., Sheng, S., Maass, N., and Sager, R. (1997a). mMaspin: The mouse homolog of a human tumor suppressor gene inhibits mammary tumor invasion and motility. *Mol. Med.* **1,** 49–59.

Zhang, M., Maass, N., Magit, D., and Sager, R. (1997b). Transactivation through Ets and Ap1 transcription sites determines the expression of the tumor suppressing gene maspin. *Cell Growth Differ.* **8,** 179–186.

Zou, Z., Anisowic, A., Hendrix, M. J., Thor, A., Neveu, M., Sheng, S., Rafidi, K., Seftor, E., and Sager, R. (1994). Identification of a novel serpin with tumor suppressing activity in human mammary epithelial cells. *Science* **263,** 526–529.

Received for publication July 20, 1999
Accepted August 6, 1999